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Cloning Sequencing and Structural Manipulation of the Enterotoxin D and E genes from Staphylococcus aureus.

Final Report

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June 1, 1990

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21702-5012

Contract No. DAMD17-86-C-6055

Kansas State University
Manhattan, Kansas 66506

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SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: Division of Biology, Kansas State University; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): Ackert Hall, Manhattan, KS 66506	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: US Army Medical Research and Development Cmd; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: Contract No. DAMD17-86-C-6055
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, MD 21702-5012	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
61102A	3M16 1102BS12	AA	119

11. TITLE (Include Security Classification): Cloning, sequencing and structural manipulation of the enterotoxin D and E genes of Staphylococcus aureus

12. PERSONAL AUTHOR(S): John J. Iandolo

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Final	FROM 11/1/85 TO 6/30/89	1990 July 1	54

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
06	13		Biotechnology, BW, Cloning, Enterotoxins, Sequencing Staphylococcus aureus, toxins, lipase, exfoliative toxin, RAI
06	13		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

We localized, cloned and sequenced the gene for staphylococcal enterotoxin D (Sed). The gene was found on a large plasmid in all strains examined. Structurally, the gene is similar to the other enterotoxins, but bears closest similarity to SEA. Transcription of sed is regulated by the agr gene product and also is dependent upon spacing of the transcription start site from the translation start. We also cloned and sequenced the genes for the exfoliative toxins A and B. Eta was localized to the chromosome and Etb was identified on a large plasmid, similar to the Sed plasmid. Deletion analysis to determine the active site resulted in finding that gene transcription of eta was dependent upon sequences near the 3' terminus of the gene. In addition, we have determined that the active site for exfoliative activity is localized to a 72 amino acid cyanogen bromide peptide at the C-terminus of the Eta molecule. We determined the mechanism by which phage conversion of lipase producing strains occurs. We found that the phage inserts into the reading frame of the structural gene and results in the production of a catalytically inactive protein.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☒ UNCLASSIFIED/UNLIMITED ☐ SAME AS RPT ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b. TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Virginia M. Miller	301-663-7325	SGRD-RMI-S

DD Form 1473, JUN 86 *Previous editions are obsolete.* SECURITY CLASSIFICATION OF THIS PAGE

FOREWORD

In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Resource Council (NIH Publication No. 86-23, Revised 1985).

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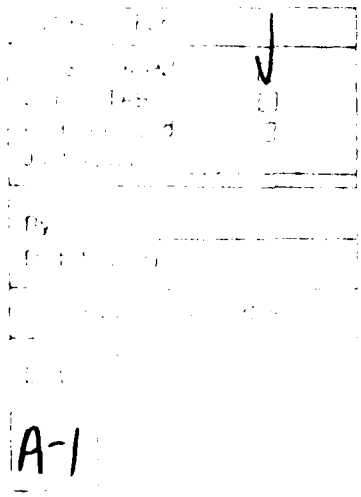

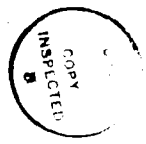

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This report represents a summary of efforts on USA Contract DAMD17-86-C-6055. It chronicles data collected over the period from November 1, 1985 through June 30, 1989. In addition to the research conducted, I wish to point out and express my gratitude to the USAMRDC not only for support but also for the vision to allow this vehicle to help train the graduate students and post-doctoral who carried out the work. This work resulted in publication of 7 papers with 3 additional manuscripts in preparation, 2 major reviews and presentation of 10 papers presented at national and international meetings.

Reference to strains, plasmids, genetic constructions, cloning and sequencing strategies and references are provided in the reprints appended to this report. This work is reported in chronological order of accomplishment.

Genetic Localization of sed:

To determine the genetic locus of sed, twelve independent Staphylococcus isolates that produced SED were obtained. Culture supernatants from these various strains were immunologically screened for toxin production by Ouchterlony analysis with anti-SED serum. In addition, plasmid DNA was isolated from each strain and examined by agarose gel electrophoresis. Each was found to contain a single 27.6 kbp plasmid. Further examination showed that they all posessed identical EcoRI restriction patterns. One strain, S. aureus 485 Pen^R (KSI1410) which contained the plasmid pIB485, was selected as representative and used for more detailed study. As a first step, we began analysis of the plasmid contained in this strain. It was presumed to

confer the resistance to penicillin and to cadmium since many large staphylococcal plasmids confer these resistances. Growth of this strain in the presence of EtBr resulted in curing of pIB485 at a frequency of 25% as determined by the loss of penicillin resistance and cadmium resistance. This $Pen^{S}Cd^{S}$ strain was designated KSI1411.

To determine if SED was still produced in KSI1411, culture supernatants from KSI1410 and KSI1411 were obtained and assayed by Ouchterlony analysis. Culture supernatant from KSI1410 again reacted with the anti-SED serum, but that from KSI1411 did not. These results indicate that either the gene encoding SED (sed) or a regulatory gene controlling SED production was located on pIB485.

In order to show that sed resided on pIB485, transformation of pIB485 into the SED^{-} strain, RN4220 was attempted. If sed was on this plasmid, transformation of pIB485 into RN4220 should convert RN4220 into an SED^{+} strain. Many attempts at this experiment were made, but they were met with little success. Transformations were performed using either penicillin or cadmium for selection, but transformed colonies were only obtained when cadmium selection was used. Plasmid analysis of these strains revealed that extensive rearrangement of pIB485 and the chromosome had occurred and resulted in the formation of four new plasmids. Two of these plasmids were shown to hybridize to ^{32}P labeled pIB485. The other two plasmids which did not hybridize to pIB485 were assumed to be of chromosomal origin. Western blot analysis of culture supernatants from these strains revealed that they did not produce SED. Presumably, pIB485 entered the cells, but instead of initiating replication,

recombinational events occurred that eliminated or inactivated sed and left the gene encoding cadmium and penicillin resistance intact. Recombinational events of this sort have been encountered several times in this lab and others (personal communications), but an explanation of this phenomenon has yet to prove satisfactory.

Since transformation experiments were not successful, other methods such as transduction and conjugation were used to introduce pIB485 into cells. Although many attempts at both methods of gene transfer were undertaken neither were successful. Several test markers were transferred directly by transduction and by conjugation to verify that the methods worked, but we never were able to obtain either penicillin or cadmium resistant tranductants or transconjugatants by mobilization due to transfer of pIB485.

Because we were uniformly unsuccessful in transferring pIB485 by any means, we re-evaluated the earlier transformation data that indicated in spite of rearrangements, pIB485 entered the recipient cells. This suggested that transformation was possible if the protoplast preparation could be stabilized. We therefore changed the composition of the regeneration from one containing sucrose, which allows protoplast growth that is similar to L-form growth. This process is unfavorable to plasmid stability and results in plasmid loss. In the presence of succinate, however, regenerating protoplasts will not divide until the cell wall is nearly or completely regenerated.

Using succinate based regeneration media (DM3) transformant colonies were obtained that were cadmium and penicillin resistant and more importantly, contained

intact pIB485. Western blot analysis of these culture supernatants showed production of SED and indicated that pIB485 converted RN4220 to an SED producing strain. This showed that sed is on pIB485 or that pIB485 contained a regulatory gene that activates a silent sed gene in RN4220. Although this latter possibility seems unlikely, we were not able to discount it at the time. Further characterization of the plasmid was carried out by restriction mapping of pIB485 was performed. pIB485 DNA was digested with EcoRI, BglII, HindIII, ClaI and the 8 possible double digest combination using these enzymes. These digestions were compared to fragments generated from E. coli phage lambda DNA digested with EcoRI and HindIII and electrophoresed in a 0.6% agarose gel. Since fragment size is directly proportional to the distance migrated in a gel, comparing the migration of the known fragment sizes of the lambda DNA to that of the migration of the pIB485 fragments allows for the calculation of the pIB485 fragment sizes. Computer generation of the pIB485 restriction map is as shown (Fig. 1). Single digestions of pIB485 using the above restriction enzymes and subsequent Southern blot hybridization analysis using fragments containing the genes encoding cadmium and the penicillin resistance region as probes enabled the pIB485 resistance markers to be mapped.

To clone the sed gene, 3-5 kbp fragments from a Sau3A partial digest of pIB485 were agarose gel purified and ligated to BamHI cut pBR322. 53 recombinant clones were obtained as determined by sensitivity to tetracycline. These clones were picked onto fresh plates and then lifted to nitrocellulose filters where they were lysed and treated with anti-SED serum and ^{125}I labeled protein A. Autoradiography of this blot

showed that five of the 53 recombinants produced a protein that cross-reacted with the anti-SED serum. Western blot analysis confirmed that the clones produced full sized and cross-reactive SED molecules. Two points of interest can be deduced from these data; 1) sed, and not a regulatory gene is contained on pIB485 and 2) sed is expressed in E. coli. The latter point is significant in light of the fact that sed and the other enterotoxin genes are expressed in the E. coli background.

To determine the insert sizes of the five positive clones, minilysates of each were prepared. Agarose gel electrophoresis of the DNA from these cells showed that they contained varying insert sizes. The plasmid containing the smallest insert (3.2 Kbp) was retained and designated pIB486. Further subcloning of sed was achieved by digesting pIB486 with EcoRI and NaeI, attaching an EcoRI linker to the blunt, NaeI end, and ligating into the EcoRI site of pUC18. This plasmid, pIB488, contains a 2.0 Kbp insert including a complete sed gene as determined by Western blotting of the cellular extract from cells containing this plasmid.

Overlapping clones for sequencing were obtained by the method described by Dale et al.(Plasmid 31: 31-40, 1985) The 2.0 Kbp insert from pIB488 was cloned into the bacteriophage sequencing vector, M13mp19. Single-stranded recombinant phage DNA was harvested, extracted, and annealed to a 20 bp oligonucleotide (RD20) that hybridizes to the EcoRI site within the multiple cloning region. The DNA was then digested with EcoRI which cleaves only within the annealed portion of the molecule. The 3' to 5' exonuclease activity of T4 DNA polymerase was then used to obtain variable deletions of the insert DNA. Poly-A tails were added using terminal Tailing

allowed recircularization of the molecule by annealing with RD20 on the other end of the fragment. Subsequent ligation was carried out with T4 DNA ligase. These ligated molecules were then transformed into E. coli JM109. Infected cells from the resulting plaques were picked and grown in L-broth. Phage DNAs from these cultures were harvested and size fractionated on a 1.0% agarose gel. DNA from the deletions selected was purified and sequenced by the dideoxy chain termination method. The opposite strand was also sequenced by going through the above process on an M13mp19 clone that had the 2.0 Kbp fragment inserted in the opposite orientation.

The DNA and derived protein sequence of the 2.0 kbp fragment is presented in Fig. 2. Analysis of the fragment revealed a large open reading frame that could encode a 258 amino acid protein with a molecular weight of 29,768. Previous amino acid analysis of the termini of the mature SED protein indicates that a serine residue is at the amino terminus. Three serine residues are present near the amino terminus of the precursor protein that could mark the amino terminus of the mature protein. By comparing the amino acid composition of the three polypeptide sequences (starting with the three serine residues) to the published amino acid composition of SED, the mature polypeptide sequence was predicted. The polypeptide starting with amino acid 30 provides the most consistent amino acid composition to that of previously published results. This polypeptide is 228 amino acids in length and has a molecular weight of 26,360 which is also in agreement with previously reported molecular weight of 27,300 daltons.

Sequence comparison of the deduced amino acid sequence of SED to that of SEA, SEB, SEC, or streptococcal pyrogenic exotoxin A (SPEA) show that there are 51.6%, 41.1%, 34.9%, and 39.2% similarity, respectively. The relatively high degree of similarity between SED and SEA was expected because SED and SEA contain similar cross-reactive antigenic determinants. However, it is notable that SED is also very similar to SPEA, a gene carried by a bacteriophage of the genus Streptcoccus. These data are taken to imply that there is a relationship among these toxins that is not necessarily reflected in their primary biological activity (emesis for the enterotoxins and rheumatic fever for SPEA), but is manifest in secondary biological features such as mitogenicity, enhancement of endotoxic shock, and immune suppression.

Localization of the sed transcription start site. The transcription start site of the sed gene was localized by S1 nuclease mapping. The results revealed the presence of a single protected fragment when using RNA isolated from S. aureus KSI1410 (Fig. 3, lane 2). The size of this fragment, determined by comparison to the simultaneously run sequencing ladder of the sed upstream region, maps the transcription start site to nucleotide (nt) -266 (Fig. 3). Just upstream from this site is a six base sequence that conforms perfectly (TATAAT, 6 of 6 matches) to the consensus -10 sequence for Bacillus sp. and E. coli promoters. Although a sequence similar to the consensus -35 sequence (TTGACA) is also present, it contains mismatches (TAGAGG, 3 out of 6 matches) and is separated from the -10 sequence by only 14 nucleotides rather than the consensus separation of 17 to 18 nucleotides.

RNA from the E. coli clone, JM109 (pIB488), gave rise to three protected fragments (Fig. 3, lane 3). These fragments identify transcription start sites at nt -266, as observed when using S. aureus RNA, and also at nts -109 and -200 (Fig. 2). Sequences upstream from both of these sites that are similar to the consensus -10 and -35 sequences were also present (Fig. 2).

In order to determine if the transcription start sites localized at nts -109 and -200 were involved in sed expression in S. aureus, a promoter deletion construct was made by digesting the sed plasmid, pIB489, with the restriction enzymes, Fnu4HI (cleavage site shown in Figure 2) and HindIII (cleaves 3' to the sed gene). This digestion isolated the sed structural gene and the promoters at nts -109 and -200 on a single DNA fragment while omitting the promoter at nt -266. This fragment was then ligated into the shuttle vector, pLI50 to form pIB476. After introduction of pIB476 into S. aureus RN4220 and E. coli JM109, the strains were assayed for SED. As shown in Figure 4a (lane 2), SED was produced by the S. aureus RN4220 strain containing the sed plasmid, pIB586 (contains all promoter sequences determined from Figure 2). However, when the promoter at nt -266 was absent, as in S. aureus RN4220 (pIB476), sed expression was reduced by 94% (Fig. 4a, lane 4). When the wild-type and deleted promoter constructs were placed in the E. coli JM109 background, both expressed the sed gene, as predicted by the S1 nuclease protection analysis (Fig. 3, lanes 2 and 3). The expression of sed observed with pIB476 in E. coli was probably the result of transcription initiation from the promoters at nts -109 and -200. Therefore, considering

the data in Figures 3 and 4, transcription of the sed gene in S. aureus appears to be initiated exclusively at nt -266.

Regulation of sed by agr/exp. The S. aureus regulatory gene, agr (also known as exp), has been shown to control the expression of a number of extracellular protein genes. The effects of the agr/exp gene product are at the transcriptional level and can either increase or decrease expression. To determine how agr/exp influences the expression of sed, pIB586 was transformed into the agr⁻/exp⁻ S. aureus strain, ISP546. The relative amount of SED made by this genetic construct (compared to that made by strain RN4220) was determined by analyzing extracellular proteins by Western blotting and densitometric scanning of the blot. These data show that the expression of sed was reduced 82% in ISP546 (Fig. 4, lane 5) compared to expression of sed in RN4220 (Fig. 4, lane 3). The reduction in the amount of SED in ISP546 is also reflected in the amount of sed-specific RNA present in this strain compared to strain RN4220 (a 91% reduction, see figure 6).

To determine if the inverted repeat located between nt -146 and nt -107 of the sed leader sequence (Fig. 2) was involved in the regulation of sed, a plasmid was constructed with this region (190 bp) deleted. The plasmid (pIB479) was transformed into S. aureus strain RN4220 and assayed for SED production. The amount of SED secreted (Fig. 5, lane 3) was reduced 49% compared to that secreted by the wild-type construct, pIB586 in the same genetic background (Fig. 5, lane 2).

The inverted repeat deletion construct (pIB479) was also transformed into ISP546 and assayed for SED production. SED made by this strain (data not shown) was

reduced by 95% compared to RN4220 (pIB586). When compared to the wild-type gene in the agr⁻/exp⁻ background, SED production was reduced an additional 48%. The additive effect of these two mutations (the agr/exp and the inverted repeat deletion mutations) suggested that the inverted repeat was involved in the regulation of sed by Agr/Exp.

To determine if the deletion in pIB479 affected the transcription and/or stability of the sed transcript, the amount of sed-specific RNA transcribed from pIB586 and pIB479 was compared. A 38% (Fig. 6) reduction in the amount of the sed transcript was observed when compared to that produced by the strain containing pIB586. This reduction in the amount of sed transcript was reflected in the amount of SED made. In addition, sed RNA levels in the agr⁻/exp⁻ strain containing pIB479 were 50% of that expressed in the wild-type strain containing pIB586 (Fig. 6). These data lent further support for the hypothesis that the inverted repeat was involved in the regulation of sed by Agr/Exp.

The 190 bp deletion in pIB479 was replaced by a heterologous DNA (i.e. lacking an inverted repeat sequence) of the same size. This plasmid, designated pIB483, was transformed into strain RN4220 and assayed for SED production. As shown in figure 5 (lane 4), this strain produced approximately the same amount of SED (determined by densitometry) as that made by strain RN4220 containing pIB586 (Fig. 5, lane 2). This implies that the reduced Sed level produced by strain RN4220 containing pIB479 is due to the length of the 5' untranslated portion of the sed transcript and not to the inverted repeat sequence itself.

Since the sequence of the sed inverted repeat is similar to the inverted repeats of the promoter regions of iron regulated genes in Gram-negative organisms (data not shown), we suspected that the sed gene might also be regulated by iron. Furthermore, if sed is an iron-regulated gene, the effects of an inverted repeat mutation would only be detected under low iron conditions. Therefore, the production of SED by KSI1410 in deferrated medium was analyzed. The amount of SED made in deferrated TSB was the same as that made in untreated TSB or in deferrated TSB supplemented with iron (data not shown). However, other proteins that cross-reacted with the anti-SED antiserum were influenced by the iron concentration. We have not had time to pursue this observation.

DNA/protein interactions. To determine if the inverted repeat could serve as a site for protein binding, the cytoplasmic proteins from strain RN450 were isolated (this strain was selected since sed was expressed as efficiently as in the wild-type strain). These proteins were passed through a DNA-cellulose column which only bound those that had the capacity to bind double-stranded DNA. The bound proteins were then serially eluted by washing the column with buffer containing increasing NaCl concentrations.

To detect DNA binding activity the fractions were analyzed using a mobility shift assay. This technique is based on the principle that a DNA/protein complex migrates slower in a 4% polyacrylamide gel than an unbound DNA molecule. As shown in Figure 7 (lanes 2 and 7), the protein fractions eluted with 0.25 M and 0.6 M NaCl contained proteins that bound to the 153 bp DNA probe that contained the inverted

repeat. In addition, a 1000-fold excess of an unlabeled DNA fragment containing the inverted repeat (Fig. 7, lanes 5 and 10) competed for binding with the labeled DNA. However, a similar unlabeled DNA fragment corresponding to the inverted repeat deletion competed for binding as efficiently as that containing the inverted repeat (Fig. 7, lane 6 and 11). These data indicate that the DNA binding proteins are not specific for the inverted repeat sequence.

Overall, these results indicate that the sed gene is regulated by the agr locus of S. aureus. However, other data suggest that additional modes of transcriptional control may affect the expression of Sed. The finding of three promoter sites utilized in E. coli suggest that some type of temporal control may exist, at least in this background. A large inverted repeat sequence which does not serve as a binding site for Agr proteins or transcripts was also investigated. This sequence which is present in several other extracellular proteins, but not in typical cytoplasmic proteins examined may also indicate that other forms of regulation may exist. However, we were unable to find any proteins that bound specifically to this region of the sed gene nor were we able to titrate specific DNA binding proteins with a genetic construct that contained only the inverted repeat region of sed. However, presently unknown temporal and/or nutritional considerations may serve to regulate expression of such binding proteins. Preliminary data suggest that the spacing of the transcription start site from the translation start has a significantly affects the stability of the messenger RNA in an as yet undetermined manner. However, investigation of these hypotheses is yet to be initiated and would depend upon continued funding.

Exfoliative Toxin

Exfoliative toxin type B is produced by bacteriophage group II strains of Staphylococcus aureus and is a causative agent of staphylococcal scalded skin syndrome. In addition to exfoliative toxin B, most isolates also produce a bacteriocin and are immune to the action of the bacteriocin. These phenotypes, as well as resistance to cadmium, were lost after elimination of a 37.5 kilobase plasmid, pRW001 from S. aureus UT0007. Transduction and transformation showed that pRW001 carried the structural genes for four phenotypic characteristics of S. aureus UT0007 (1) exfoliative toxin B production, (2) bacteriocin production, (3) bacteriocin immunity, and (4) resistance to $Cd(NO_3)_2$. These data represented the first successful transduction and transformation of the S. aureus phage group II exfoliative toxin plasmid in the phage group III genetic background.

The exfoliative toxin B structural gene (etb) was identified by shotgun cloning a HindIII digest of the plasmid pRW001 into the 5.5 kb HindIII fragment of the gram positive/gram negative shuttle plasmid pDH5060. The ligation mixture was transformed into E. coli LE392 selecting for ampicillin resistance carried on pDH5060. This step facilitated isolation of large quantities of recombinant plasmid DNA which was needed for transformation of S. aureus. To ensure equal selection for all fragment sizes of pRW001 cloned into pDH5060, growth on several plates was pooled to represent the entire clone library. Plasmid DNA was then isolated from the mixed clones and used to transform S. aureus 4220 by selection of the chloramphenicol resistance marker of pDH5060. The staphylococcal clone bank was then screened for

various markers contained on pRW001. The cadmium resistance gene was isolated on a clone that contained a 2.0 kbp HindIII fragment of pRW001, the etb gene was isolated on a 1.7 kbp fragment and the region containing the bacteriocin synthesis and resistance genes was isolated on a 7.0 kbp PstI fragment of pRW001. A restriction map of the pRW001 was constructed from these data and is presented in Fig. 8.

During the isolation of the toxin gene we observed that etb was not expressed in E. coli. Neither the supernatant fluids nor lysates showed the toxin positive phenotype even though gel electrophoresis showed that the full length clone was present. We therefore subcloned the toxin containing gene fragment into the selection-expression vector pSCC31. This positioned the etb gene downstream from the phage lambda P_L promoter. This construction was then transformed into E. coli and tested for exfoliative toxin B production by Western blot analysis. In this configuration, exfoliative toxin B was produced. The inability to express etb from its own promoter in E. coli is similar to the result originally obtained with the enterotoxin B gene but is in stark contrast to the expression of other staphylococcal genes in the gram negative genetic background. This suggests that at least two types of promoters exist in S. aureus.

The 1.7 kb HindIII restriction fragment of DNA containing etb was isolated from plasmid pIJ002 and cloned into the replicative form of bacteriophage M13mp18 and mp19 DNA and transformed into E. coli JM103. Single stranded DNA was isolated from the bacteriophage produced by the transfected cells. This DNA was then sequenced by the dideoxy chain termination method of Sanger using [α-^{35}S]dATP instead of ^{32}P.

The sequence was analyzed by computer and shown to contain an open reading frame (ORF) that compared favorably with the predicted amino acid analysis of exfoliative toxin B (ETB). A likely methionine initiation codon was found at position 181 within range of a suitable ribosome binding site. The ORF which begins at 181 is 822 bases in length and terminates at position 1000. Translation of this ORF identified 22 of the 26 N-terminal amino acid residues (N-terminus = Lysine) as well as the C-terminal residue (Lysine) of ETB which were determined by chemical sequencing methods. A 31 amino acid signal peptide precedes the toxin molecule with an alanine residue at the proposed cleavage site, where processing of the precursor occurs to yield the mature protein.

Shortly after these sequence data were published, several errors were discovered which required redetermination of the sequence shown in Figure 9. Additional chemical sequence data obtained from Drs. Schmidt, Spero and Johnson-Winegar at USAMRIID agrees totally with the protein derived from the corrected DNA sequence and provides an important internal control of the sequence data. The new derived protein sequence data indicates that the ETB molecule is 277 amino acid residues and has a 31 residue signal peptide which when cleaved leaves a mature protein of 246 residues. The molecular weights of the species are 30,769 for the precursor and 27,318 for the mature protein. Furthermore, there is complete agreement between the DNA derived sequence and the chemically determined sequence for the first 40 amino acids and between the first 48 amino acids of a cyanogen bromide derived peptide of ETB beginning at residue 172 and continuing

through residue 219. The composition of the protein is interesting in that there is neither tryptophane or cysteine present and the molecule lacks the cysteine loop found in the enterotoxins. The transcription signals originally reported remain unchanged and show -35 and -10 promoter sequences, a ribosome binding site and a transcription stop signal that closely approximates the canonical sequences.

Following these studies we began cloning of the eta gene. This element is chromosomal so a diffferent strategy was utilized to isolate it. Bulk chromosomal DNA prepared from S. aureus UT0002 was used to construct a bacteriophage λgt11 library. The plaques (40,000) were screened with rabbit antiserum that had been extensively adsorbed with lysates of the E. coli host strain. Plaques binding antibody were identified with ^{125}I-labeled Protein A and exfoliative toxin A (ETA) production was confirmed by Western blot. Five clones were found that reacted positively with the antiserum. One of these phage was randomly selected for further study. The DNA insert isolated from this phage was approximately 3.2 kilobases and was recloned into the shuttle vector pLI50 and transformed into E. coli LE392. Immunoblots confirmed that this plasmid contained the eta structural gene which was expressed and biologically active in E. coli.

Deletion analysis further localized the gene to a 1391 bp fragment. This fragment was sequenced by the Sanger dideoxy method and is presented in Fig. 10. The G+C content of eta is 31% and is typical of the S. aureus genome. However, the G+C content of the 150 bp sequence upstream from the methionine start codon (nucleotide 313) was even lower in G+C content (19%), suggesting that the region could serve as

the potential binding site for RNA pouymerase to initiate transcrition. A potential -35 sequence and a -10 sequence that could serve as promoter regions were identified. Furthermore, the probable ATG translation start site is preceded by the sequence GGATGA, which qualifies as a potential ribosome binding site. A potential transcription stop codon is located at position 1154 and is followed 79 bp downstream by a stem-loop structure at positions 1232-1259.

Translation of the ORF yielded a 280 amino acid polypeptide that corresponded to the published properties of ETA. A 38 amino acid signal peptide precedes the N-terminus of the mature ETA protein which is cleaved immediately after the sequence Ala-Lys-Ala of residues 36-38. Removal of the signal peptide results in a mature ETA protein containing 242 amino acid residues with a molecular weight of 26,950. The sequence was identical to that of peptides of ETA that were determined by automated Edman degradation. A contrasting finding was that the C-terminal amino acid is glutamic acid rather than lysine. These results were substantiated independently by O'Toole and Foster (J. Bacteriol. 169: 3910-3915. 1987).

The amino acid composition of the DNA derived mature protein sequences of ETA and ETB were compared to the chemically derived values. The data are quite similar and confirm that ETA has a single tryptophane and methionine residue and the lack of cysteine. ETB was found to lack cysteine and tryptophane as already indicated. Comparison of the amino acid composition of both ETA and ETB indicates that they are reasonably similar proteins that are rich in polar amino acids.

Direct comparison of the protein sequences of ETA and ETB is shown in Fig. 11. Three prominent regions of similarity are evident in which the match was extensive. The first occurred in the N-terminal portion of the molecule at positions 46 - 70 (20 of 25 residues match, 80%), the second near the middle at positions 106 - 134 (17 of 29 residues match , 58%) and the third near the C-terminus at positions 201 through 221 (17 of 21 residues match, 81%). No other regions of significant similarity were present. The total number of amino acids matched by computer alignment was 110 (45%) out of an average of 245 residues. This extensive similarity might not have been predicted because of the lack of antigenic relationship between the toxins. However, when the relative hydropathicities are compared, it was clear that much of the sequence of each toxin represents highly preserved domains in which the amino acid differences are fairly conservative. We interpret this to indicate that folding of the two proteins is similar, so that the sites of biological activity, presumably focused at the regions of sequence homology, can be similarly presented to the appropriate substrate.

Prerequisite to studies of the mode of action of ETA and ETB, we attempted to determine the sites of biological activity within the molecules. We hypothesized that the C-terminal region of the proteins was important for biological activity. This is a region where 81% of the amino acids of ETA and ETB (17 of 21 residues at positions 201-221) are identical. Moreover, preliminary evidence in our laboratory showed that a cyanogen bromide derived peptide of ETA containing the 72 C-terminal amino acid residues which includes the region of greatest similarity, caused exfoliation in neonatal mice (unpublished data).

Deletions of eta cloned in pJJ825, were made using Exonuclease III and Mung Bean Nuclease. Three derivatives with deletions of 203, 278, and 591 bp were selected for further experimentation. The inserts were cloned into pUC18 for analysis in gram negatives and pLI50 for analysis in gram positive backgrounds. The constructs were transformed into E. coli JM109, E. coli SG23006 Lon$^-$ and into S. aureus RN4220.

Using computer modeling, we determined that the molecular weights of the ETA proteins produced from the deleted Eta genes should be 13,887; 29,458; and 32,222, respectively. These data were obtained by alignment of the junction sequences of the eta inserts to the sequence of PUC18. This enabled us to determine the extent of translational read-through before encountering a termination signal in pUC18. We failed to detect any ETA on a Western blot from the deleted constructs in E. coli JM109.

One hypothesis regarding the absence of ETA production from the JM109 plasmid containing strains, was that the proteins produced from the deleted clones were unstable. Perhaps unstable, deleted proteins were rapidly degraded by E. coli proteases. This hypothesis was rejected by analyzing results of ETA production in the Lon$^-$ strain SG23006. As was true for the deleted constructs in JM109, only the isolate containing the wild type plasmid produced ETA. ETA was not produced from the deleted clones in the Lon$^-$ strain, SG23006.

Similar results were obtained with the deleted genes in S. aureus. This was true in spite of the fact that in the E. coli strains, transcription was initiated from the lac Z promoter and in the S.aureus strains, transcription signals from eta were utilized.

ETA was not produced by those strains containing deletions in eta of 591 and 278 bp, respectively. KSI865, the isolate containing a 203 bp deletion in eta, produced a small quantity of ETA in comparison to KSI866, the wild type S. aureus clone. These data suggest that the 3' region of the eta gene is important for message and/or protein stability. The junction between the eta inserts and pLI50 was not determined, so we did not know the expected sizes of the proteins as we did for the E. coli pUC18 constructs.

RNA was isolated from our S. aureus clones and analyzed by a slot blot to estimate the stabilities of the mRNA transcripts. The results showed that mRNA was not produced from KSI863 lending support to the hypothesis that the 3' region of eta which was deleted in this clone is important for message stability. The same conclusion may also be implied for KSI864 although this isolate produced a small amount of mRNA. pJJ864 contains an additional 313 bp in the 3' region of eta which is not present in pJJ863. Perhaps the nucleotides in this area are important for increased mRNA stability. KSI865, the isolate containing only a 203 bp deletion, produced a significant amount of mRNA, almost equal to that of the wild type, KSI866, but ETA production was only a fraction of the wild type level. Again, the finding suggests that the 3' region of eta is important for increased message stability. When 278 bp are deleted from the 3' area, transcription seems to be greatly affected, but it is only slightly affected when 203 bp are eliminated. The specific role of these additional 75 nucleotides is unknown; the sequence of this region reveals no obvious secondary structure which may be involved in transcription or translation.

Because KSI865 produced a significant quantity of mRNA, we expected it to produce a significant quantity of protein. This was not the case. Only a small amount of ETA was detectable. The eta insert in pJJ865 contains only 10 amino acid residues fewer than full-length ETA. Nevertheless, our data suggest that the region coding for these 10 amino acids is necessary for complete and effective translation. How the 3' region of eta is involved in message and/or protein stability is unknown.

The results of the neonatal mouse assay paralleled the Western blot data. If we were unable to detect ETA from the deleted clones on an SDS-PAGE gel, then it was unlikely to expect a positive Nikolsky sign in mice injected with toxin samples from these clones. Only the mouse injected with whole cells of S. aureus containing the wild type gene showed flaking and peeling of the epidermis. E. coli strains containing containing the wild type gene did not show exfoliation. It is possible that the E. coli strains do not produce enough toxin to be detected biologically. Another possibility may be that the E. coli inoculum does not survive well in the mouse due to host defenses. Confirmation of either explanation necessitates further research.

From mRNA analysis, we expected to see exfoliation in the mouse injected with cells harboring the smallest deletion of eta. A significant amount of message was produced by this clone (although we do not know its molecular size). However, as this strain produced little protein, the mouse result was not surprising. Further research will be required to determine the exact site of biological activity within the ETA molecule. It will be necessary to discover more precisely the role of the 3' region of eta in transcription and translation. Northern blot analysis would be useful for

determining the sizes of mRNA produced. Such data would reveal whether the messages produced from more extensively deleted genes were intact or degraded. This information would be important in addressing the question of mRNA stability.

Regulation of the Lipase Gene (geh)

Many strains of staphylococci produce a true lipase or glycerol ester hydrolase (EC 3.1.1.3). The activity of the staphylococcal lipase gene is negatively regulated by bacteriophage lysogenization, also known as lysogenic conversion. We have reported cloning the lipase gene (geh, for glycerol ester hydrolase) and shown that the mechanism of conversion is most likely due to the interruption of the gene by insertion of the phage DNA. To characterize this unique regulation of gene expression, we sequenced the lipase gene and determined the phage insertion site by Southern hybridization analyses.

The nucleotide sequence of the entire 2,968 bp DNA sequence containing the geh gene has only a single large reading frame extending from nucleotide 706 to 2776 that represents the probable coding region for lipase (Fig. 12). Evidence supporting this conclusion is threefold. (1) A Shine-Dalgarno sequence which is complementary to the 3'end of E. coli 16S rRNA was found 4 nucleotides upstream of the probable ATG initiation codon. (2) BAL 31 deletion mutagenesis identified this region as being required for lipase activity. Data from SDS-PAGE indicated that lipase is a protein with a molecular weight of about 70,000 daltons, which agrees closely with the molecular weight of the protein deduced from the DNA sequence.

The region upstream from the proposed start codon contains a very high proportion of A+T resulting in many possible binding sites for RNA polymerase. In fact, several potential sequences similar to E. coli promoters were found but no attempt was made to determine which promoter was used in transcribing the lipase mRNA.

We found no cysteine residues in the deduced protein sequence. This finding is in contradiction to earlier indication of others that thiol blocking agents inhibit lipase activity. We cannot explain this discrepancy, but the sequence data are compelling to suggest that the earlier observation was in error. A possible reason for the lack of cysteine is that proteins with a low level or lacking cysteine are more flexible molecules and may pass readily through the rigid cell wall. For example, we reported that enterotoxin B which contains a single disulfide loop is sequestered within the cell wall and passes slowly into the extracellular medium. Furthermore, in the case of lipase, the lack of cysteine may also allow conformational changes that are necessary for enzymatic activity when a water soluble enzyme reacts with a hydrophobic lipid.

Staphylococcal phage L54a inserts into the ClaI D frgament (corresponding to nucleotides 2197-2968) of the geh containing DNA fragment. We used restriction mapping to further localize the insertion site and to determine whether it lies within the structural gene of lipase. The ClaI D fragment was isolated and cloned into the AccI site of the replicative form DNA of bacteriophage M13 mp18. This placed the right end of the ClaI D fragment adjacent to the PstI site of M13 mp18. After linearization of the plasmid with PstI, the ClaI D fragment was digested with Bal 31 exonuclease

to generate serial deletions from the right end of the fragment (i.e. nucleotide 2776), while the left end was protected by M13 sequence. The deleted molecules were then blunt end ligated and transformed inot E. coli JM103. A set of deletions were obtained that corresponded to the removal of about 80, 170, 270 and 360 bp from the right end of the fragment. These plasmids were used as probes in Southern hybridization of genomic digests of the phage lysogenized strain PS54. If the probe made with the deleted fragment contained the insertion site, it should identify the two bands representing phage-chromosome junction fragments in ClaI or PstI digested DNA (both enzymes cleave the bacteriophage genome at least once) of the lysogenic strain. On the other hand, if the probe made with deleted ClaI fragment D did not contain the insertion site due to BAL 31 digestion it should identify only one junction fragment. Probes with either 80 or 170 bp deleted from the right end of the ClaI D fragment identified both junction fragments. On the other hand, probes with 270 or 360 bp deleted from the right end of the fragment identified only one junction (left junction) fragment. However, on longer exposure, a faint band corresponding to the right junction fragment was detected in the 270 bp deletion but not in the 360 bp deletion. This result implied that bacteriophage L54a DNA inserted into the ClaI D fragment between 270 and 360 base pairs from the right end of the element. Thus, L54a inserted into the structural gene of lipase near the carboxyl end of the protein between amino acids 635 and 664.

It is interesting that hydropathicity data indicated that the region from amino acid 35 to 310 is predominantly hydrophilic, whereas hydrophobic residues were found

in the region from residue 310 to the carboxyl end. Since lipase is an enzyme whose substrate is hydrophobic we would predict that the hydrphobic region from amino acid 310 to the C terminus is assoicated with the active site of the enzyme. This conclusion is supported by 1) a deletion in plasmid pLI233 which has about 20 amino acids deleted from the C-terminal end and is lipase negative. Up to 500 bp could be removed from the left end of the fragment by BAL 31 digestion and up to 80 bp could be removed from the right end without influencing activity. Larger deletions at either end of the insert resulted in loss of enzymatic activity (Fig 13) .2) the finding that the phage L54a insertion site is near the carboxyl end of the molecule and results in the production of a truncated lipase protein that has no catalytic activity and 3) the finding that extensive homology exists in this region with lipase cloned from other species of staphylococci (Fig. 14). Therefore, the region either contains the active site or is important for the conformation of the active site.

We cloned and sequenced fragments of the S. aureus PS54 lipase gene which contained the chromosome/bacteriophage junction fragments (attL and attR). These fragments were in turn used to probe a 4.5 kb ClaI frament of the bacteriophage L54a genome that contained the phage attachment site (attP).

The sequence of the att sites (Fig. 15) reveals an 18 bp core sequence common to all four regions. This feature is similar to the bacteriophage lambda att sites in which the common core is a 15 bp sequence. Unlike the common core of the lambda att sites which have an 80% A+T content, the core of the L54a att sites is only 61 percent A+T. The A+T content of the DNA flanking the core region (the arms) which extends from

-50 to +50 is 63% in the attP site and 55% in the attB site. In view of the fact that the percent A+T of the S. aureus genome and staphylococcal phages is 62-70%, the percent A+T found in the core sequence and the surrounding region is not untypical.

Also indicated in Fig. 15 are regions of dyad symmetry, inverted repeats and direct repeats. These probably represent possible binding sites for proteins that mediate the recombination. No attempt was made to confirm the protein binding capacity of these regions. However, inasmuch as there are unaltered tandem direct repeats of the core sequence flanking the prophage as a result of integration, the crossover point must occur within the core sequence. Furthermore, the flanking core sequences also suggest that recombination occurs via staggered cuts, and that recombination is not only site-specific but also orientation-specific.

In every reported system of site-specific recombination, the gene encoding the enzyme which mediates the recombination reaction is located near the recombination site, so our initial approach to identify the gene or genes responsible for L54a recombination (recombinase gene) was centered on the DNA near the attP site. Two DNA fragments containing attP were cut from the bacteriophage genome. One (4.5 Kb) contained attP with DNA extending rightward from it (ClaI restriction fragment, see Fig. 16) and the other (3.5 Kb) contained attP with DNA extending leftward from it (PvuII-HindIII restriction fragment, see Fig. 16), These fragments were cloned into a shuttle vector pLI50 and the plasmids designated pLI451 and pLI475. They were transformed into protoplasts of S. aureus RN4220 and the presence of recombinase activity was tested by assaying for lipase activity. RN4220(pLI461) had no lipase

activity, whereas RN4220(pLI475) remained lipase positive. The recombinase expressed in RN4220(pLI461) mediated recombination between attP on plasmid pLI461 and attB on the lipase gene of the RN4220 chromosome showing the recombinase gene is located within the segment of DNA rightward from the attP site.

More precise mapping of the recombinase gene function was achieved by cloning and testing recombination activity of sequential deletions of the 4.5 Kb ClaI fragment. The results of this experiment are schematically shown in Fig. 16 indicating the size of the deletion along with an indication of the effect of the deletion on integration. These data indicate that one end of the recombinase gene is located to the left of the EcoRV site (about 2 Kb rightward to the attP site). In addition, since there is no promoter in the vector preceeding the cloning site, the 2 Kb fragment must contain the promoter of the recombinase gene.

Our results also suggest the functioning of more than one gene and is supported by the following argument. Our assay for integration is the loss of lipase activity consequent to insertion. However, after prolonged incubation of strain PS54 lipase activity could be detected at a frequency of 10^{-4} to 10^{-3} due to loss of the prophage. The same phenomenon was observed with transformants carrying the cloned attachment sites. The plasmids pLI461, pLI462 and pLI463 would also convert these cultures to the lipase negative phenotype at a frequency of 10^{-4}. Consequently, this implies that the excision gene is also located within the same 2.1 Kb cloned DNA fragment as the gene for integration. It is posible that in L54a, two enzymes are responsible for recombination and are located within the short 2.1 Kb DNA segment.

Confirmation that the lipase negative phenotype was due to integration of the plasmid containing the attP site and the recombinase gene was obtained from Southern hybridizaton analyses. ClaI digested bulk chromosomal DNA prepared from transformants of various plasmids was hybridized to a probe of the 770 bp ClaI-D fragment of the lipase gene which contains the attB site. Since ClaI cleaves each of the plasmids at least once but does not cleave the ClaI-D fragment of the lipase gene, the probe would identify two bands if integration occurred, whereas it would identify only one band if there was no integration. Fig. 17 shows the results of this analysis which confirm that integration has occurred.

Lysogenization of L54a in many strains of S. aureus results in loss of lipase activity caused by insertion of the prophage genome at the 3' or carboxyl end of the lipase structural gene which is *essential for* catalysis. This indicates that a truncated catalytically inactive lipase protein deleted by 46 amino acids should be produced by the lysogenized strain. Indeed, preliminary immunological screening indicated that the lysogenized strain does produce a cross reactive material that lacks lipase activity. By examining the nucleotide sequence of the left junction of the bacterial chromosome and L54a DNA (i.e., attL), a stop codon TAA was found adjacent to the core sequence. The sequence analyses, therefore, support the mechanism of the lysogenic conversion of the lipase.

The importance of this work is as a model for the regulation of the ß-toxin of Staphylococcus which is also mediated by phage conversion. This activity however, is governed by both positive and negative conversion. There are two converting

phages that mediate the expression of β-toxin. One of these is a negative regulator of expression similar to the L54a system, but the second is both a positive and negative converting phage. This second phage carries the staphylokinase gene and upon lysogenization confers staphylokinase activity and inactivates β-toxin activity in the host cell. Some of the interesting question to ask are whether the insertion sites of this phage and L54a are similar and within the structural β-toxin gene, whether these phages are similar to or have given rise to the phages that carry the sea and spea genes and to the elements that harbor other extracellular toxins of Staphylococcus? Many other interesting questions relating not only to toxigenesis but also to the basic biology of phage conversion in this system can be asked.

Identification of a lipase transcriptional regulatory locus

We have developed a simple and sensitive quantitative assay for measurement of lipase activity. Most published assays require complicated and expensive chromatographic analysis to determine the amount of fatty acid hydrolysed from triglycerides. However, this simple assay can be used for lipase determinations in agar plates or in spectrophotometric analysis which improves precision. The assay is based on the fact that triglycerides are insoluble in water solutions, but β-glycerides are soluble. Lipase cleaves end fatty acids from triglycerides liberating β-glyceride. We therefore, emulsified 0.25% tributyrin in a solution of 0.1% carboxymethylcellulose (added to stabilize the emulsion) by sonication for 7 - 10 minutes at 50w. This suspension was used to analyze lipase activity in solutions of lipase to standardize the

reaction kinetics and from filter sterilized culture supernatants. Reduction of optical density at 450nm was measured. The reaction produced a linear decrease in O.D. whose slope was indicative of lipase activity. Reaction velocity also varied linearly with dilution of enzyme concentration. The reaction was specific in that no activity was observed with lipase negative strains that produced other lipolytic enzymes (phospholipases C and D). In addition, sufficient sensitivity exists in the assay to measure activity differences in transcriptionally regulated lipase mutant strains.

Use of this assay allowed characterization of a trans-active lipase regulatory element. We identified this novel regulatory locus by transposon mutagenesis using Tn551. An insert designated Ω1058 which maps to the right of thy in segment A on the standard staphylococcal chromosome map was found to significantly (>90%) depress the expression of geh (lipase gene) which maps in segment E. Because these two loci are roughly 180° degrees apart on the circular chromosome, it is almost certain that Ω1058 has inactivated a trans-active positive regulatory element. We have been able to transduce the mutation into other Lip$^+$ strains and reproduce the mutant phenotype. We have determined that this mutation in the original mutant strain and in the transductant strains simultaneously down-regulates hly and hlb, the α and β toxin genes and seb (the enterotoxin B gene) and coa (the coagulase gene). This effect is substantially different from the effect of the Agr mutation which has no effect on geh, down-regulates hly, hlb, and seb but up-regulates coa. Further, restriction mapping of the chromosome of S. aureus has shown that agr and Ω1058 map in different locations. Therefore this appears to be a separate regulatory locus. We have cloned

the Tn551 insertion and used this clone to reprobe the wild type chromosome. The wild type sequences have also been cloned onto the cosmid pLAFR3 on a 25 kbp DNA fragment. Present efforts are designed to subclone and identify the specific regulatory locus on this fragment.

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2. Gill, S.R. Genetic analysis of extracellular protein synthesis in Staphylococcus aureus. Ph.D. dissertation. 1989.

3. Bayles, K. W. Genetic and Molecular analysis of the gene encoding staphylococcal enterotoxin D. Ph.D. dissertation 1989.

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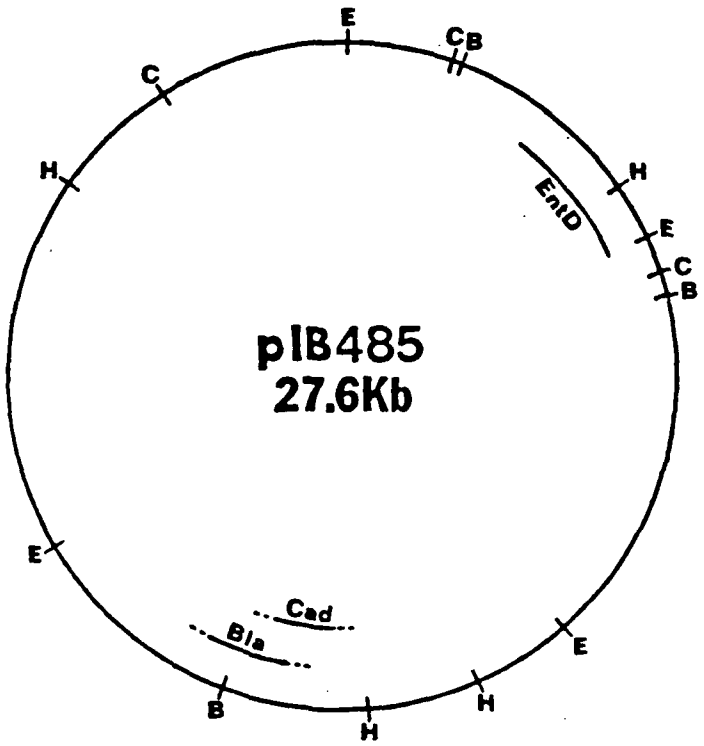


Fig. 1 . Physical map of pIB485. Restriction sites were mapped by restriction digest analysis of pIB485. Restriction enzymes used were EcoR1 (E), HindIII (H), BglII (B), and ClaI (C). Genes encoding beta-lactamase and cadmium resistance are designated bla and cad, respectively.

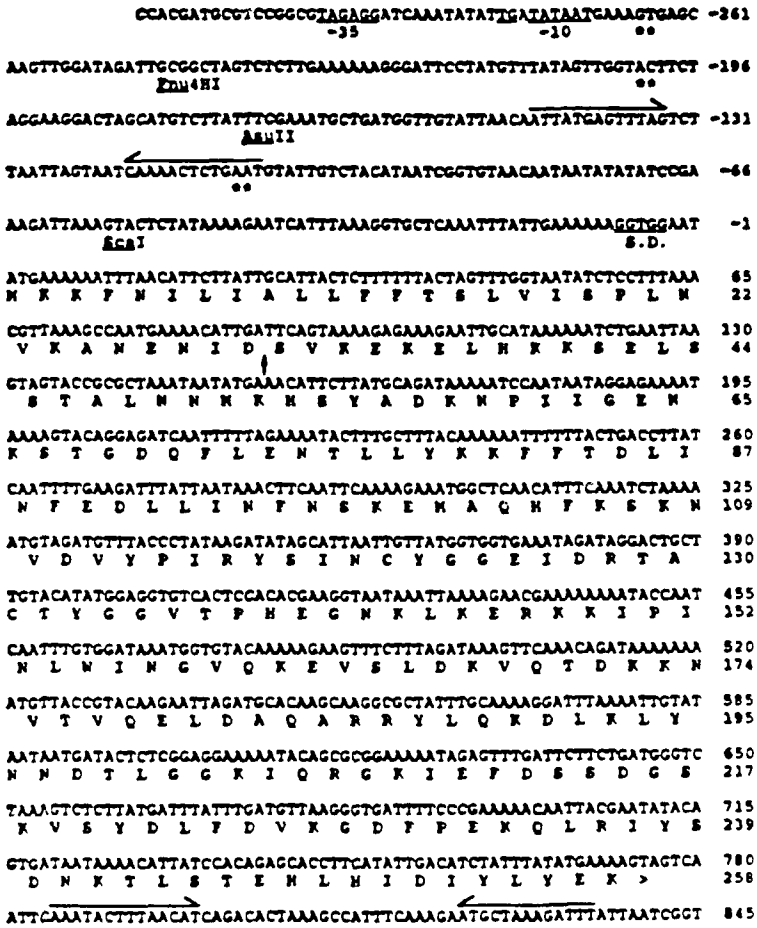


Fig. 2 . Sequence analysis of the entD gene. Shown is the nucleotide sequence of the entD open reading frame which encodes a 258 amino acid protein. The deduced amino acid sequence is also presented. The leader peptide cleavage site is indicated by a vertical arrow. The horizontal arrows near the 3' end of the entD gene indicate a possible transcription termination signal.

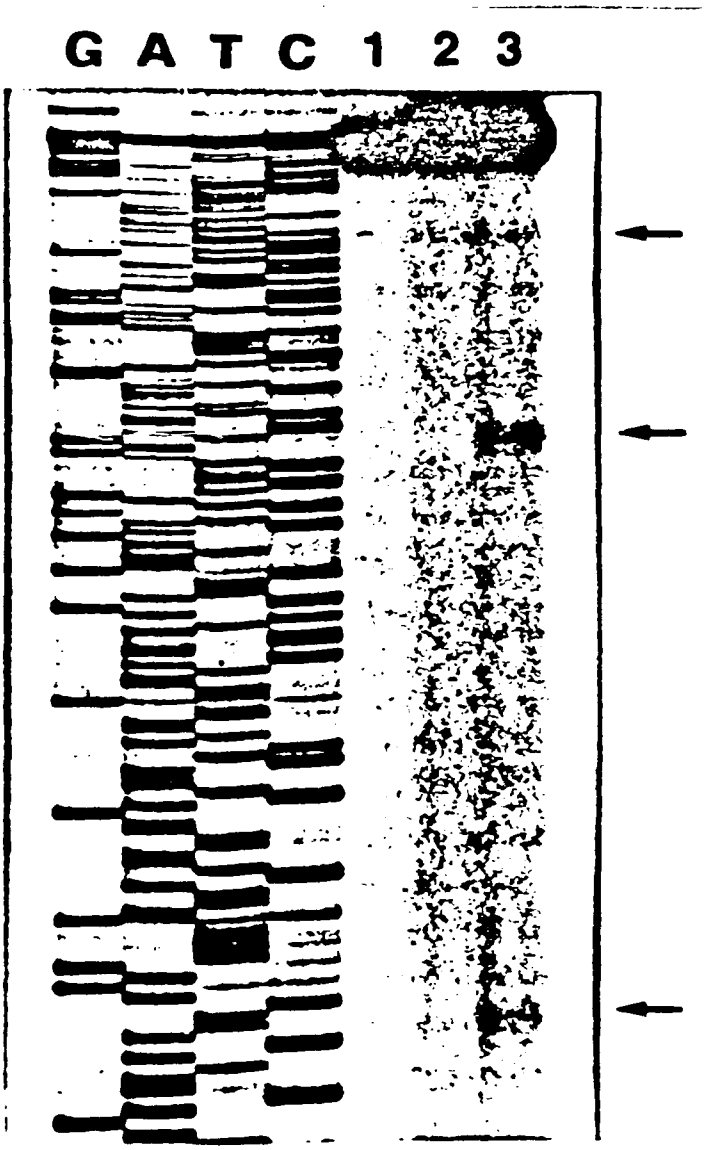


Fig. 3. Analysis of the transcription start site of the gntD gene. Total RNA was isolated from S. aureus KSI1410 (lane 2) and E. coli KSI1454 (lane 3) and used to hybridize to ^{32}P labeled fragments containing the upstream sequences of gntD. A hybridization was also carried out using no protecting RNA (lane 1). The samples were digested with S1 nuclease and seperated on a sequencing gel. Protected fragments were detected by autoradiography.

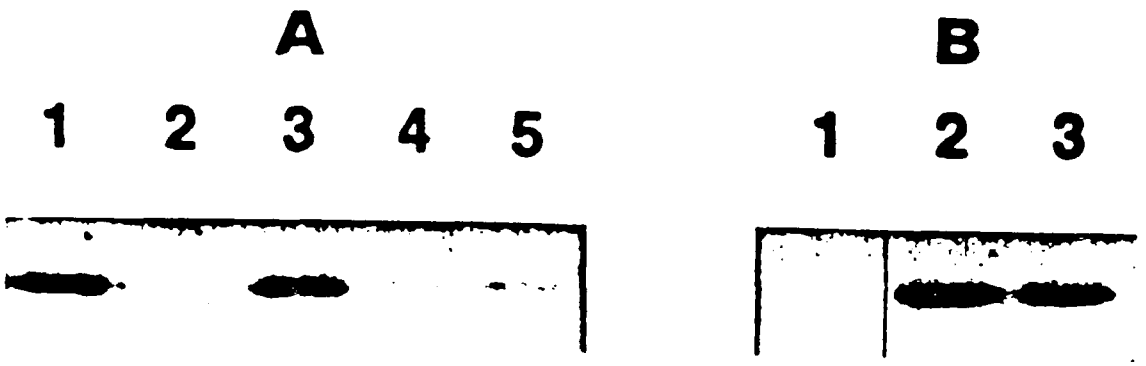


Fig. 4 . Western immunoblot analysis of the expression of entD in S. aureus and E. coli strains containing entD plasmid constructs. a) Equal amounts of extracellular proteins from 18 h cultures of the S. aureus strains. Lanes: 1, partially purified SED (100 μgs); 2, RN4220 (pLI50); 3, RN4220 (pIB586); 4, RN4220 (pIB476); and 5, ISP546 (pIB586). b) Total proteins from 4 h cultures of the E. coli strains. Lanes: 1, JM109 (pLI50); 2, JM109 (pIB586); and 3, JM109 (pIB476). SED was detected using anti-SED antisera and ^{125}I labeled protein A.

Fig. 5. Western immunoblot analysis of entD mutants in S. aureus. Equal amounts of extracellular proteins from 18-h cultures were seperated in a 15% polyacrylamide gel and blotted to nitrocellulose. Lanes: 1, partially purified SED (100 μg); 2, RN4220 (pIB586); 3, RN4220 (pIB479); and 4, RN4220 (pIB483). SED was detected using anti-SED antisera and ^{125}I labeled protein A.

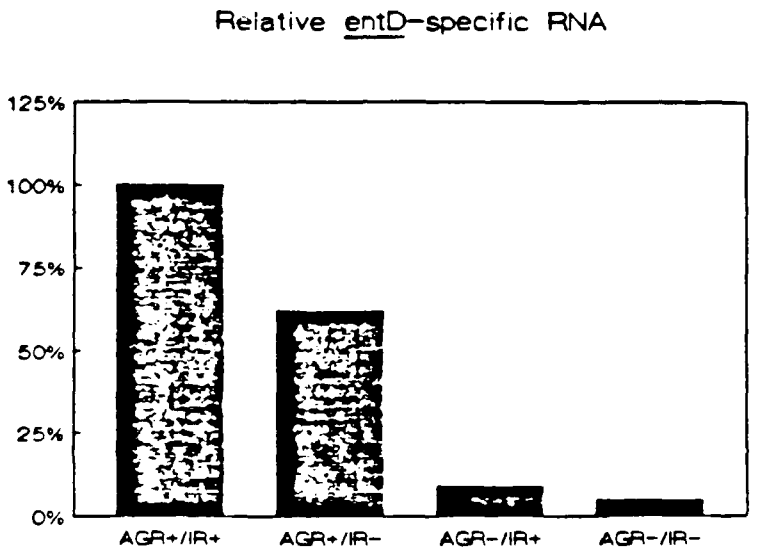


Fig. 6. The relative amount of entD-specific RNA made in mutants of S. aureus. Equal amounts of total RNA from RN4220 (pIB586) (AGR+/IR+), RN4220 (pIB479) (AGR+/IR-), ISP546 (pIB586) (AGR-/IR+), and ISP546 (pIB479) (AGR-/IR-) were added to GeneScreen Plus in a slot blot apparatus and hybridized to the ^{32}P-labeled entD containing DNA insert in pIB479. The amount of probe hybridizing was detected by autoradiography and quantitated by densitometric scanning of the autoradiogram.

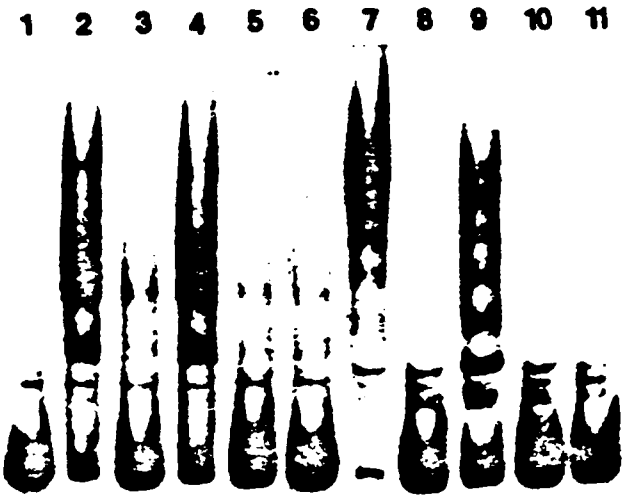


Fig. 7. Gel-retardation assay of the 0.25 M NaCl (lanes 2-6) and 0.6 M NaCl (lanes 7-11) protein fractions eluted from a DNA-cellulose column (see materials and methods). DNA fragments containing the inverted repeat were end-labeled with ^{32}P-dATP and incubated in the presence of a 1000 fold molar excess of unlabeled DNA fragments containing the inverted repeat (lanes 3 and 8); a 100 fold molar excess of unlabeled DNA fragments containing the inverted repeat (lanes 4 and 9); a 1000 fold molar excess of unlabeled 1.3 Kbp DNA insert from pIB586 (contains inverted repeat, lanes 5 and 10); and a 1000 fold molar excess of unlabeled 1.1 Kbp DNA insert from pIB479 (has inverted repeat region deleted, lanes 6 and 11). Lanes 2 and 7 contain DNA samples incubated in the absence of competitor DNA and lane 1 contains a DNA sample incubated in the absence of protein extract. The DNA/ protein mixtures were seperated in a 4% non-denaturing polyacrylamide gel and visualized by autoradiography.

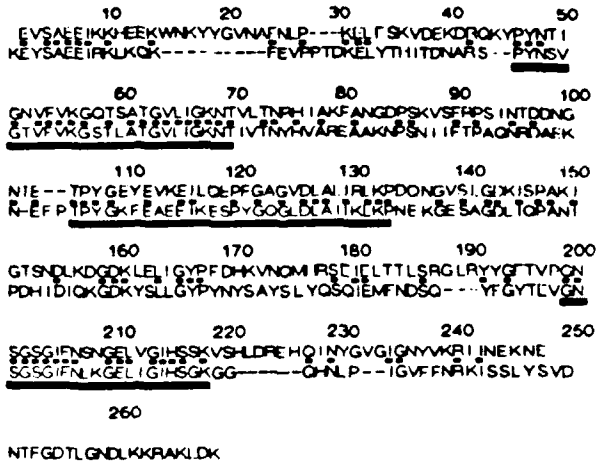


FIG. 11 Comparison of the amino acid sequences of ETA (top row) and ETB (bottom row). Sequence identities are indicated by bars, and dashed lines indicate gaps introduced to produce the optimal alignment. Numbering includes gaps and does not correspond to the residue number obtained from the DNA sequence. Alignment was constructed by computer using the algorithm of Wilbur and Lipman (30) with a K-tuple of 1, window of 20, and gap penalty of 1. Three regions of substantial homology are underlined.

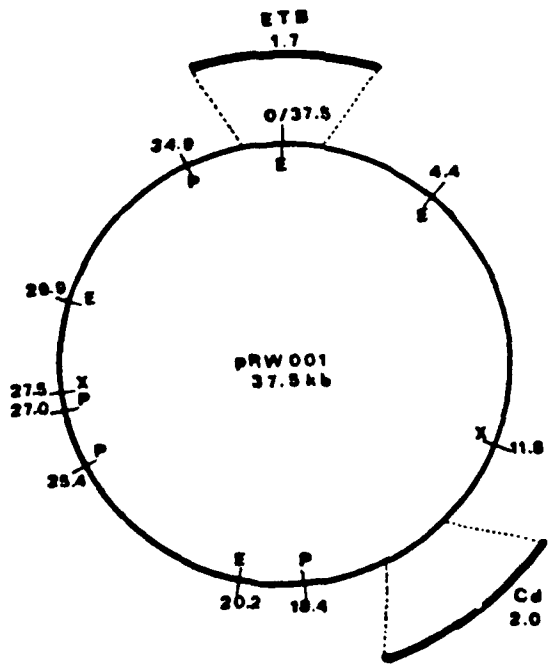


FIG. 8 Restriction endonuclease map of plasmid pRW001 derived by digestion with *Eco*RI (E), *Pst*I (P), and *Xho*I (X). Map distances are expressed in kilobases. The *etb*$^+$ and *cad*$^+$ loci were determined by Southern hybridization analyses with the purified *Hin*dIII fragments from the expressing clones as probes. The *Hin*dIII sites marking the ends of these two probes are represented by dotted lines. Note that the bacteriocin genes were tentatively assigned to the 7.0-kb *Pst*I fragment (coordinates 18.4 to 25.4).

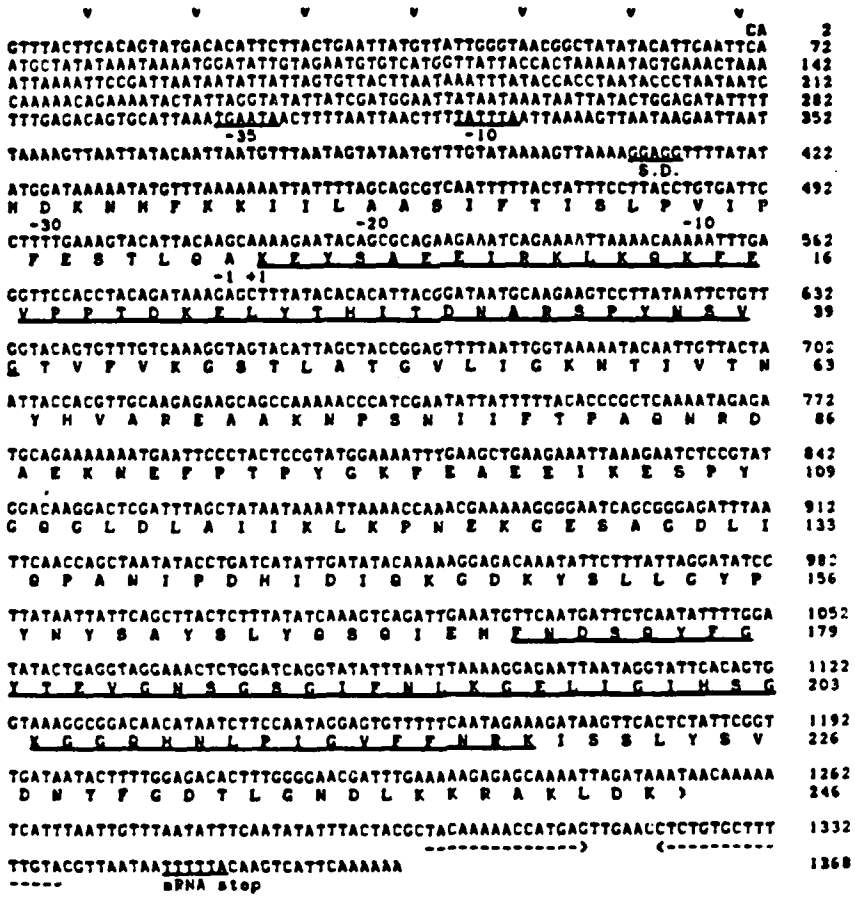


FIG. 9 Sequence of the 1,368-bp DNA fragment containing the *etb* gene and sequence of ETB derived from it. The locations of the presumptive −35, −10, Shine-Dalgarno ribosome binding site (SD), −1, +1 protease processing site, stem-loop termination structure (facing arrows), the stop codon (>), the mRNA transcription stop site, and the chemically derived peptide sequence (underlined) are indicated.

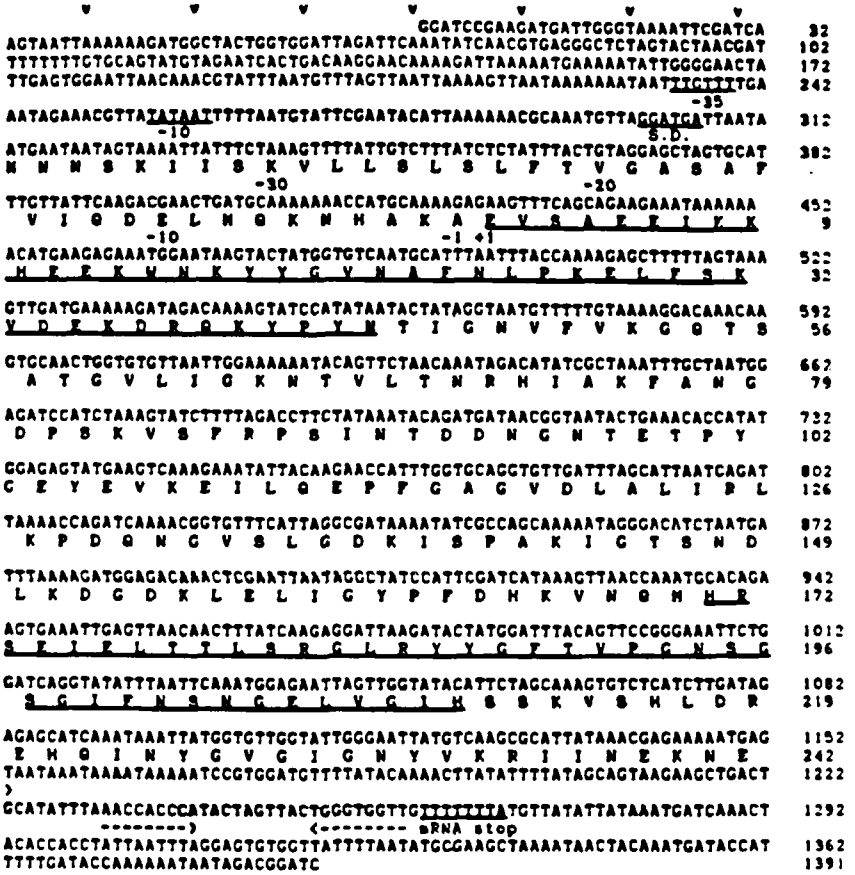


FIG. 10 Sequence of the 1,391-bp DNA fragment containing the *eta* gene and sequence of ETA derived from it. The locations of th presumptive −35, −10, Shine-Dalgarno ribosome binding site (SD), −1, +1 protease processing site, stem-loop termination structure (facir arrows), the stop codon (>), the mRNA transcription stop site, and the chemically derived peptide sequence (underlined) are indicated.

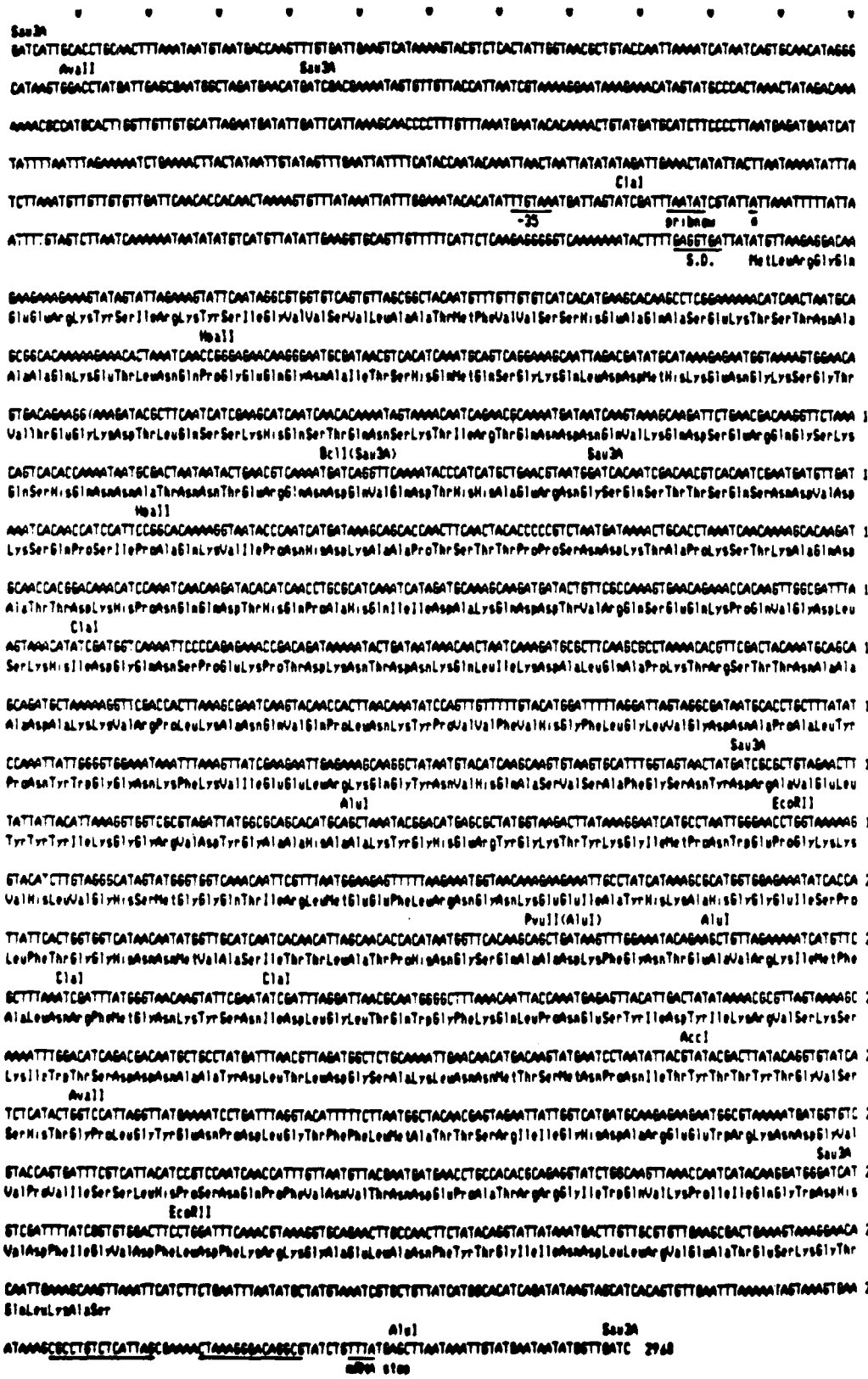


FIG. 12 Nucleotide sequence of the 2,968-bp DNA fragment containing the *geh* gene and the predicted amino acid sequence of the lipase. The potential promotor region (−35 and pribnow) , the possible start site for transcription (°), the Shine-Dalgarno sequence (S.D.) , and the possible mRNA stop site (arrows marking the inverted repeats for the potential stem-loop) are indicated.

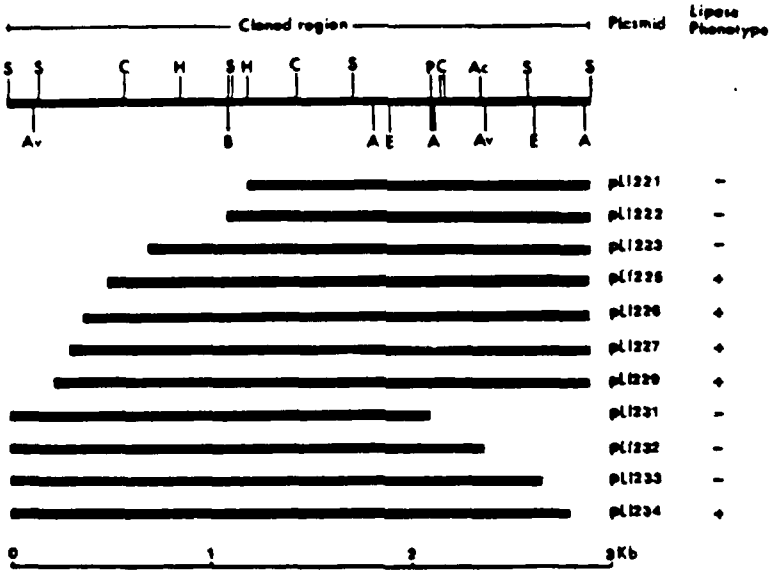
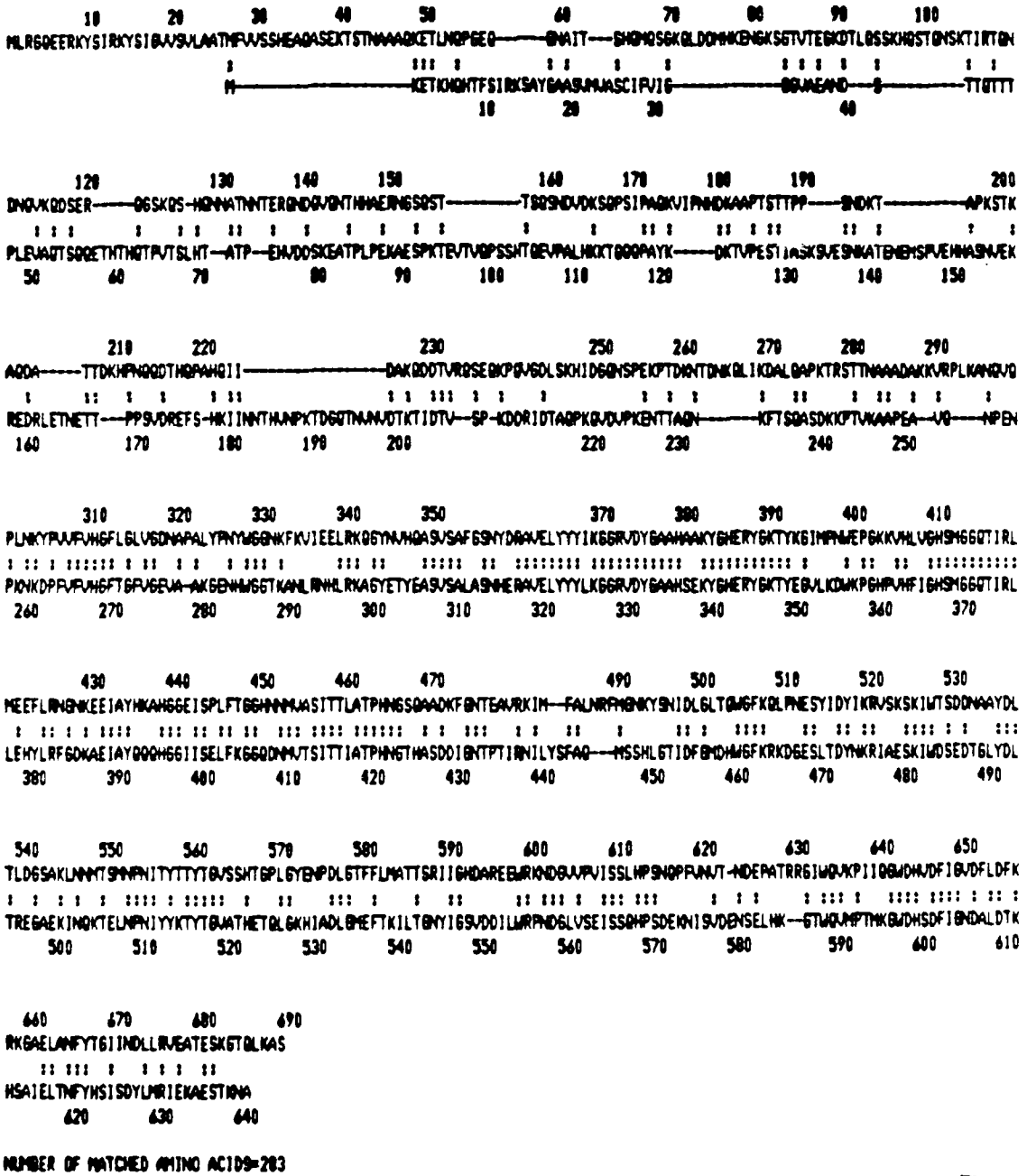


FIG. 13 Restriction endonuclease map of the 2,968-kb lipase-containing DNA fragment and the deleted fragments used to map the location of the *geh* gene. Only the cloned regions are shown. A, *Alu*I; Ac, *Acc*I; Av, *Ava*II; B, *Bcl*I; C, *Cla*I; E, *Eco*RII; H, *Hpa*II; P, *Pvu*II; S, *Sau*3AI. Restriction sites were determined from the DNA sequence. Lipase activity is indicated by + and −.



NUMBER OF MATCHED AMINO ACIDS=283

FIG. 14 Comparison of the amino acid sequence of lipases from *S. aureus* PS54C (top line) and *S. hyicus* (bottom line). The sequences were aligned with a computer by the method of Wilbur and Lipman Gaps were introduced to obtain maximum homology. The default parameters set were as follows: K-tuple, 1; window size, 20; gap penalty, 1. Colons between the amino acids of the two sequences indicate matches. The single-letter designation of amino acids is used.

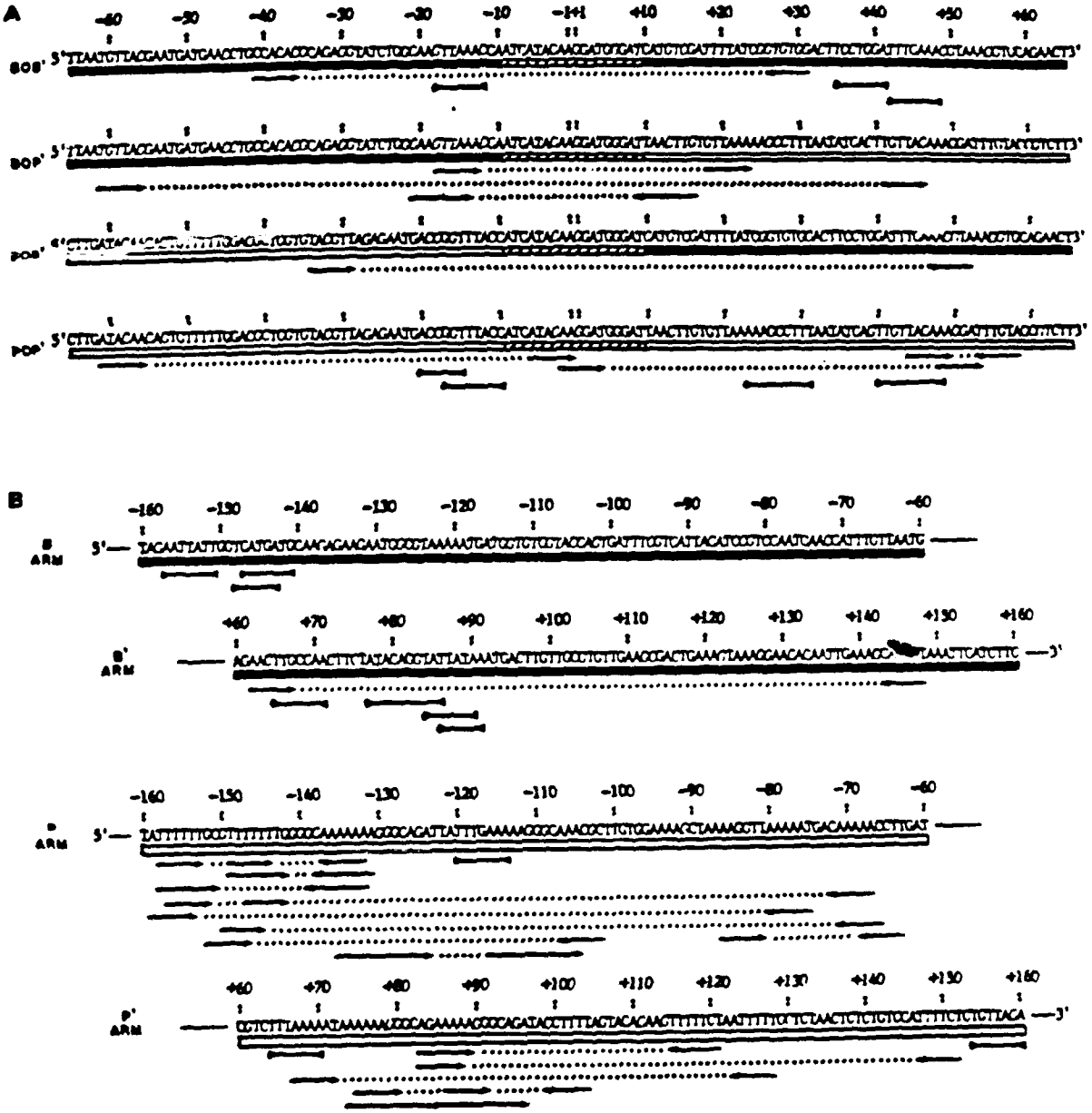
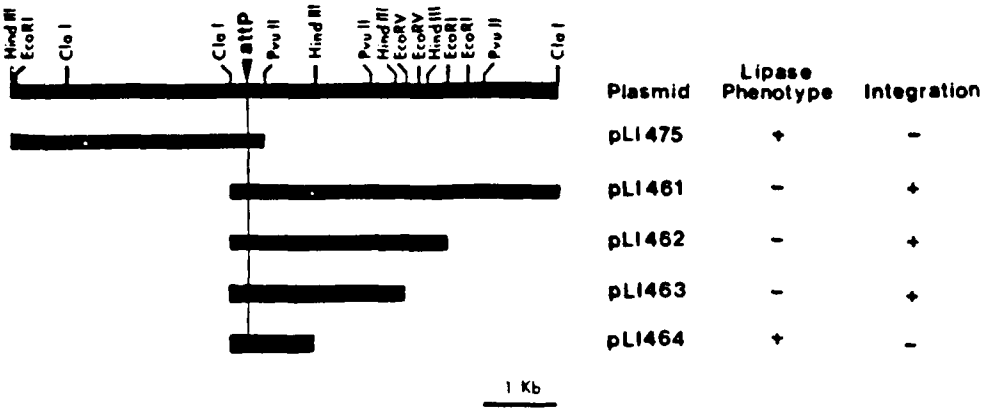


FIG15 Nucleotide sequences of the regions containing the *att* sites. Sequences are numbered from the center of the core; the b immediately to the right is +1 and the base immediately to the left is −1. (*A*) The central 130 bp of each of the four *att* sites that encompas 65 bp on each side. (*B*) Distal portions of the four arms extending 100 bases leftward from −60 and 100 bases rightward from +60. Nomenclat shown at left of each region (BOB', etc.) is adapted from that of the bacteriophage λ system (24). Solid bars represent bacterial DNA; op bars, LS4a DNA; hatched bars, core sequences. Molecular palindromes (▷◁), inverted repeats (→ ←), and direct repeats (→ →) are indicat Direct repeats are omitted in *B*, except one set, found in the P and P' arm, that is of special importance and is discussed in the text. Dot lines connect the pairs of repeats. The criteria used in marking the sequence features are as follows: minimum of 6 bp with no mismatches inverted repeats and direct repeats; a single or no central mismatch with at least 3 bp to each side, or two central mismatches with at leas bp to each side, in molecular palindromes.



Plasmid	Lipase Phenotype	Integration
pLI475	+	-
pLI461	-	+
pLI462	-	+
pLI463	-	+
pLI464	+	-

FIG. 16 Localization of the recombinase gene near the *attP* site. Relevant restriction sites are indicated. Arrowhead indicates the approximate *attP* site. Vertical line indicates the approximate location of the core sequences. Lipase phenotype of transformants generated by transforming the various deleted plasmids is indicated. Integration as determined by lipase activity is also indicated.

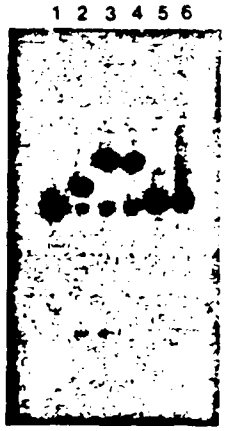


FIG. 17. Southern hybridization analysis of integration. DNA from the transformants containing the deleted plasmids was digested with *Cla* I, subjected to electrophoresis in agarose, blotted to nitrocellulose, and hybridized with ^{32}P-labeled probe prepared from the *Cla* I fragment D containing the *attB* site (i.e., the DNA fragment from base pair −430 to +340 of Fig 1A). Digested DNA was from RN4220 (lane 1), RN4220(pLI461) (lane 2), RN4220(pLI462) (lane 3), RN4220(pLI463) (lane 4), RN4220-(pLI464) (lane 5), and RN4220-(pLI475) (lane 6).

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